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***In vivo* transmission studies
of ,*Candidatus Mycoplasma turicensis*
in the domestic cat**

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1 Summary

The natural transmission routes of the three feline hemotropic mycoplasmas, *Mycoplasma haemofelis*, '*Candidatus Mycoplasma haemominutum*', and '*Candidatus Mycoplasma turicensis*' (CMt), are largely unknown. Since saliva of CMt-infected cats has been found to be PCR-positive, we hypothesized that direct transmission via social or aggressive contact may occur. The aim of our study was to evaluate these transmission routes. For this purpose, five SPF cats received CMt-positive saliva or blood inoculi subcutaneously to mimic cat bites whilst another five cats received the inoculi orally (blood)/oronasally (saliva) to mimic social contact. Blood samples were monitored for CMt infection using quantitative real-time PCR and for seroconversion by a novel Western blot assay. Neither oral nor subcutaneous inoculation of CMt-positive saliva led to CMt infection as determined by PCR in the recipient cats independent of the presence of immunosuppression. When infectious blood containing the same CMt dose was given subcutaneously, four of the five cats became PCR-positive, while none of the five cats inoculated orally with up to 500 µl of infectious blood became PCR-positive. Subsequently, the latter cats were successfully subcutaneously infected with blood. All CMt exposed cats seroconverted. In conclusion, CMt transmission by social contact seems highly unlikely, whilst transmission via aggressive interaction (cat bites) may occur if the recipient cat is exposed to infectious blood.

2 Introduction

Three hemotropic mycoplasmas, the causative agents of feline infectious anemia, have been differentiated in cats: *Mycoplasma haemofelis* (Mhf), previously ascribed to the genus *Haemobartonella*, '*Candidatus M. haemominutum*' (CMhm) and '*Candidatus M. turicensis*' (CMt) (10, 32, 34, 37, 46). Another hemotropic mycoplasma, similar to the canine hemotropic mycoplasma '*Candidatus M. haematoparvum*', has also been suggested to occur in cats (38). Their pathogenic potential varies. Immunosuppression or preexisting retroviral infections may potentiate the severity of anemia (2, 14, 17, 36, 46).

The diagnosis of feline hemotropic mycoplasmas (aka hemoplasmas) had been limited because microscopic detection of the agents on blood smears was unreliable (40, 45). Recently, sensitive TaqMan real-time PCR assays have been introduced and are now considered to be the gold standard for detection and differentiation of all three species (42, 45, 46). Using PCR, a worldwide distribution of feline hemotropic mycoplasma infections in felids has been documented (3, 12, 27, 29, 30, 40, 41, 44, 49). So far, no commercial routine serologic assay for feline hemoplasmas is available. Serodiagnostics have depended on crude Mhf antigens, either purified from the blood of experimentally infected cats or present on blood smears from acutely infected cats, as a substrate for indirect fluorescent antibody assays (1, 9). Results of immunoblotting and immunofluorescence assays using antigenically undefined blood extracts showed that anti-Mhf antibodies were detectable two to three weeks post infection (p.i.) (1, 9). Lately, an immunodominant protein of *Mycoplasma suis*, the porcine hemoplasma species, was identified as HspA1, a surface-localized dnaK-analogous protein by serological proteome analysis and screening of a genomic *M. suis* library (23-25).

The natural mode of transmission of feline hemoplasmas has been under investigation for many years but is still controversial. Transmission by

experimental blood inoculation via the intraperitoneal, intravenous and oral routes has been successful (8). In addition, transplacental and lactogenic transmission have been discussed (6, 16, 20). However, these studies had been performed prior to the introduction of specific molecular assays. Blood transfusions have been reported to be effective in hemoplasma transmission (13, 19, 45). Furthermore, blood-sucking arthropods might be involved in the transmission of feline hemoplasmas in certain regions. Unfed ticks in Japan but not in Switzerland were found to be PCR-positive for feline hemoplasmas (39, 47). The cat flea *Ctenocephalides felis* is also thought to be a vector but transmission has not been proven (50, 51). Moreover, the low prevalence of feline hemoplasmas in fleas and ticks in Switzerland contradicted the hypothesis of arthropods being the major vector at least in this geographical region (47). Because of the close phylogenetic relationship of CMt and rodent hemotropic mycoplasmas (*M. coccoides* and *M. haemomuris*) an interspecies transmission of hemoplasmas between rodents and cats was suggested but could not be proven so far (46, 47).

Most recently, the investigation of shedding patterns by real-time PCR showed CMt shedding in saliva and feces (4, 47) and we hypothesized that feline hemoplasmas could be transmitted by direct contact (social or aggressive) via saliva (47). Our hypothesis is supported by an increased occurrence of hemoplasma infections in male cats and cats with a history of cat bite abscesses (17, 21, 30, 40, 45).

The aim of the present study was to address this hypothesis and investigate the possible direct transmission of CMt between cats. To mimic aggressive contact and cat bites, saliva or blood samples from CMt-infected cats were inoculated subcutaneously to recipient cats, whilst social contact between cats was simulated by oronasal and oral inoculation. All cats were monitored after CMt exposure and blood samples were assessed for CMt infection using sensitive real-time PCR and a novel Western blot assay.

3 Materials and Methods

3.1 Cats

Thirteen specific pathogen free (SPF) cats (Liberty Research Inc., Waverly, NY, USA) were included in the study. They were kept in a confined university facility in groups under etiologically and hygienically ideal conditions. Prior to the start of the experiment, each cat was clinically examined and blood, conjunctival, oropharyngeal, and rectal swabs were collected from all cats to verify their SPF status. The cats were found to be negative when the samples were tested for the three feline hemoplasmas, feline coronavirus, feline calicivirus, feline herpesvirus-1, *Chlamydomphila felis*, feline parvovirus, *Bartonella henselae*, feline leukemia virus, and feline immunodeficiency virus by real-time TaqMan PCR or reverse transcription-PCR and by serology for antibodies to the feline corona virus as previously reported (18, 22, 26, 28, 31, 33, 35, 43, 45). Cats were blood typed by a commercial gel column technique for determination of the feline blood types A, B and AB (ID-Gel Test Feline A+B Typing; DiaMed AG, Cressier sur Morat, Switzerland): 11 cats were of blood-type A and two cats had blood-type B. Of the 13 cats, three adult male castrated cats (two and three years of age), were infected intraperitoneally with CMt to obtain infectious saliva and blood samples (amplificatory cats, Cats X, Y, and Z). Ten juvenile cats (two months of age), housed in two groups of five cats each, were used as the recipient cats for the transmission studies.

3.2 Clinical examination, sample and inoculi collection

Clinical examination was performed by a veterinarian at least twice a week and cats were controlled daily by the animal keeper. Complete hemograms of the amplificatory cats were performed prior to immunosuppression and prior to CMt infection, daily during saliva collection and sporadically until 167 days p.i.

for Cats X and Y and until 119 days p.i. for Cat Z. Saliva swabs (Primella, Migros Genossenschafts-Bund, Zurich, Switzerland) of the amplificatory cats were collected every day once the cats had turned blood PCR-positive by placing swabs sublingually and in the cheek pouches. For collection of large volumes of saliva for the transmission study, saliva production was stimulated by a subcutaneous injection of neostigmine (Valeant Pharmaceuticals, Birsfelden, Switzerland; 0.5 mg/kg) 10 minutes prior to ketamine/midazolam narcosis (ketamine 20 mg/kg and midazolam 0.1 mg/kg, i.m.; Vétoquinol, Ittigen, Switzerland/Roche, Reinach, Switzerland). Saliva samples were divided into aliquots for infection and PCR. Saliva was collected in 2 ml syringes, placed in Eppendorf tubes and divided into aliquots for infection and PCR.

EDTA-anticoagulated blood samples and serum were collected regularly from the ten recipient cats prior to and regularly after CMt exposures for PCR analysis, hematology and Western blot analysis. Body temperature and body weight were recorded at each sampling date. For humane reasons, cats were given supportive care (infusions, analgesics) when required.

3.3 Hematology and biochemistry

Hemograms consisting of packed cell volume (PCV), hemoglobin (Hb), erythrocyte count, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV) and total leukocyte count were performed using a Cell-Dyn 3500 (Abbott, Baar, Switzerland). PCV values between 33 % and 45 % (5% and 95% quantiles, reference range of the Clinical Laboratory) were considered to be within the normal range; anemia was defined as a PCV value below 33 %. Modified Wright-stained blood smears were prepared from fresh EDTA-anticoagulated blood using an AMES Hema Tek slide stainer (Bayer, Zurich, Switzerland), Diff-Quick[®] (Medion Diagnostics, Düringen, Switzerland) stained smears were

prepared from EDTA-anticoagulated and native blood. All smears were microscopically evaluated for CMt. A serum biochemistry profile was performed if clinically indicated using a Cobas Integra 700 instrument (Roche Diagnostics, Rotkreuz, Switzerland).

3.4 Intraperitoneal infection

To obtain fresh infectious saliva and blood samples, the three amplificatory cats were infected by intraperitoneal administration of CMt as described previously (7). Cats X, Y and Z received 9×10^5 copies of CMt in 0.9 to 1 ml of heparinized blood preserved in 20 % DMSO (Sigma-Aldrich, Buchs, Switzerland) at -80°C. All three cats were immunocompromised by two intramuscular administrations of methylprednisolone acetate (10 mg/kg, Depo-Medrol ad us. vet., Pfizer AG, Zurich, Switzerland): Cats X and Y were injected 16 and 9 days prior to CMt inoculation; Cat Z received methylprednisolone 8 and 1 days prior to CMt inoculation. Additionally, Cat Z received prednisolone orally (4 mg/kg/d, Streuli Pharma AG, Uznach, Switzerland) for 20 days starting 14 days p.i..

During Cats X, Y, and Z's peak bacteremia 40 ml of blood and 2 ml of bone marrow were collected. Bone marrow aspirates were obtained from the proximal humerus under general anesthesia (ketamine and midazolame) or acepromazine 0.1 mg/kg and propofol (induction 5 mg/kg; maintenance 1 mg/kg when required (Arovet AG, Zollikon, Switzerland/Fresenius KABI Ag, Stans, Switzerland)) and analgesia (buprenorphine, 0.01 mg/kg, Essex Chemie, Luzern, Switzerland). The bone marrow aspiration needle (Rosenthal type, 16 G x 2", Tyco Healthcare Group LP, Mansfield, U.S.A.) was introduced through a small skin incision, the muscle and the bone cortex into the bone.

Subsequently the three amplificatory cats underwent antibiotic treatment. In Cats X and Y, antibiotic treatment with marbofloxacin (Vétoquinol, Ittigen, Switzerland) was started at 2 mg/kg q24h for a total of 10 days 41 days p.i..

Afterwards, marbofloxacin was replaced by doxycycline (Grünenthal GmbH, Mitlödi, Switzerland) 5 mg/kg q12h for a total of 14 days. Cat Z was treated with doxycycline 10 mg/kg q24h for 14 days starting at day 41 p.i..

3.5 Oronasal and subcutaneous exposure to CMt DNA containing saliva

As a model for direct transmission via saliva and to simulate aggressive and social contact, saliva from the CMt-positive amplificatory cats was inoculated orally or subcutaneously into the recipient cats within 20 minutes of collection. Five recipient cats (group A) were exposed oronasally to saliva (Table 1, A I.). The cats each received a cumulative dose of 2 ml of saliva containing 8×10^2 copies of CMt over 19 days. An aliquot of 100 µl of saliva containing 1×10^2 copies of CMt was inoculated subcutaneously to each of the five recipient cats of group B (Table 1, B I.).

When inoculations A I. and B I. were unsuccessful, the cats were immunosuppressed orally with prednisolone prior to and during the next CMt inoculation as indicated in Table 1 (A II. and B II.). Development of saliva-associated abscesses necessitated termination of immunosuppression in group B, and amoxicillin-clavulanate treatment (Synulox, 25 mg/kg, q12h for 9 days, Pfizer AG) in Cat 7.

3.6 Oral and subcutaneous exposure to CMt containing blood

Because the salivary inoculations with or without immunosuppression did not lead to CMt infection in the recipient cats as determined by real-time PCR, cats of both groups A and B were then inoculated with infectious blood (Table 1, A III. and B III.). The blood aliquots used for inoculation contained the same copy number administered previously via saliva (Table 1, A III. and B III.).

Each cat of group A ingested 63 μ l of blood containing 8×10^3 copies of CMt voluntarily by licking (Table 1, A III.). For this group, the monitoring period was prolonged to two months as an incubation period of up to 51 days for oral transmission was described by Flint (7). However since the cats remained PCR-negative despite this prolonged monitoring period, they were subsequently inoculated with a larger blood volume of 500 μ l (Table 1, A IV.). When the cats of group A remained PCR-negative after oral and oronasal exposures to saliva, they were challenged subcutaneously with 50 μ l of CMt-positive blood (Table 1, A V.) in order to confirm that the cats could be infected with CMt. To simplify subcutaneous injection each aliquot of infectious blood was diluted in heparinplasma of the same cat prior to infection to a total volume of 100 μ l.

Group B cats received 1×10^3 CMt copies in 10 μ l of blood subcutaneously (Table 1, B III.). One cat, Cat 7, that remained PCR-negative, was inoculated again in exactly the same way 103 days after the first subcutaneous inoculation (Table 1, B IV.).

3.7 TNA extraction

Total nucleic acids (TNA) were extracted from 100 μ l of EDTA-anticoagulated blood using the MagNa Pure LC Total Nucleic Acid Isolation Kit I (Roche Diagnostics). Negative controls consisting of 200 μ l phosphate buffered saline were prepared with each batch. The DNA was eluted in 100 μ l of elution buffer. Saliva samples and the saliva swabs in PBS were heated at 40°C for 10 minutes, centrifuged twice at 6,600 x g for 1 minute before TNA was extracted with a MagNa Pure LC total nucleic acid isolation kit (Roche Diagnostics) as previously described (15).

3.8 Quantitative real-time PCR assay

All TNA samples were analyzed by real-time TaqMan PCR for the presence of CMt DNA as described previously (46). Water was used as a negative control. Positive control samples were included in each run. For absolute quantification, all positive blood samples were reanalyzed with a plasmid standard containing the cloned 16S rRNA gene of CMt (46) diluted tenfold.

3.9 Serology

A recombinant HspA1 protein of Mhf was expressed in *E. coli*. The partial sequence of the HspA1 protein of Mhf was obtained using consensus primers derived from the HspA1 nucleotide sequence of *M. suis* (#AM265536) and other mycoplasmas (*M. penetrans*, *M. pneumoniae*). The 899 bp-fragment was ligated into the pQE-30 Xa Vector (Qiagen, Hombrechtikon, Switzerland), which was then transformed into *E. coli* Top10 (Invitrogen, Basel, Switzerland). Protein production, SDS-PAGE and immunoblotting were performed as described previously (23) with the following modifications. The expression was induced by 1 mM IPTG. Recombinant *E. coli* cultures were grown in LB medium supplemented with 100 µg/ml ampicillin. The 6xHis-tagged protein was purified by nickel affinity chromatography (Qiagen, Hombrechtikon, Switzerland) or chelating sepharose (GE Health care biosciences, Uppsala, Sweden). 10 µg of the antigen were loaded per well in the gel and feline serology samples were incubated at a dilution of 1:100. Immunoreactive proteins were visualized using horseradish peroxidase-labeled goat anti-cat IgG (Sigma Aldrich) as secondary antibodies and 4-chloro-1-naphtol as the chromogenic reagent.

The Western blot was evaluated with samples from four SPF cats infected with Mhf (6 weeks p.i.), CMhm (21 weeks p.i.) or CMt (8 weeks p.i.), and from 18 hemoplasma-free SPF cats. To study seroconversion after CMt infection, the

three amplificatory and the ten recipient cats were tested prior to and at various time-points after CMt exposure.

3.10 Statistics

Data were compiled in Excel (Microsoft) and statistical analyses were performed using the Excel add-in Analyse-it (Analyse-it Software, Leeds, UK) and the Graph-Pad Prism (GraphPad Software, San Diego, CA, USA). For correlation analyses the Spearman rank correlation test was used. CMt blood loads, PCV values and the duration between CMt exposure and the first PCR-positive result were analyzed for significant differences among three groups of cats using the Kruskal-Wallis test (pKW) and the Dunn's multiple comparison post-test. Analyses between two groups were conducted using the Mann-Whitney U-Test (pMWU). A p-value <0.05 was considered to be significant.

4 Results

4.1 Characteristics of cats infected intraperitoneally with CMt

All three amplificatory cats inoculated intraperitoneally with CMt became infected. DNA of CMt was detected in blood samples, salivary swabs and bone marrow collected from all three cats (Fig. 1). All extraction and the negative PCR controls of the entire study tested PCR-negative. Cats X and Z became blood PCR-positive at the first sampling date, 6 and 4 days p.i. respectively, Cat Y became blood PCR-positive at the sampling on day 11 p.i. (Fig. 1). Peak CMt blood loads reached 10^6 copies/ml of blood whilst bone marrow loads reached 10^4 copies/ml in all three cats. The cats remained PCR-positive in the blood until treatment (see below). Cats X, Y, and Z became anemic with minimum PCVs of 23%, 29%, and 21% at 18, 19, and 11 days p.i., respectively (Fig. 1A, B, and C). When the combined data from the three cats were analyzed, hemoplasma loads were negatively correlated with PCV values ($r = -0.35$; $p = 0.0057$), the erythrocyte counts ($r = -0.42$; $p = 0.0008$) and the hemoglobin values ($r = -0.32$; $p = 0.0139$). This was mainly due to a strong correlation found in Cat Z. When the data from latter cat were analyzed individually, the following inverse correlations with CMt load were found: PCV ($r = -0.63$; $p = 0.0028$), erythrocyte count ($r = -0.70$; $p = 0.0006$), hemoglobin ($r = -0.66$; $p = 0.0016$). Significant correlations, or trends towards significance, were found in some cats for other erythrocyte indices and high hemoplasma loads: high MCH (Cat X: $r = 0.60$; $p = 0.0054$) and MCV values (Cat X: $r = 0.43$; $p = 0.0559$; Cat Z: $r = -0.57$; $p = 0.0092$), but the indices were never found to go beyond reference limits. Mild inappetence and apathy were observed in all three cats two to three weeks p.i..

4.2 CMt shedding via saliva

PCR-positive results were obtained for 15 of 22 salivary swabs collected from Cat X, for 11 of 19 salivary swabs collected from Cat Y and for 16 of 26 salivary swabs collected from Cat Z (Fig. 1). The CMt loads in PCR-positive saliva samples ranged from 1×10^2 to 6×10^3 copies per ml of saliva (median: 7×10^2 copies/ml).

4.3 Transmission experiment: oronasal and subcutaneous exposure to CMt DNA containing saliva

No evidence of CMt infection as determined by real-time PCR was detected in any of the recipient cats after attempted transmission of CMt via saliva (up to 7×10^3 copies oronasally and 1×10^3 copies subcutaneous, respectively). Results were identical regardless of the presence or absence of preexisting immunosuppression in the ten cats (Table 2, A I. and II. and B I. and II.).

4.4 Transmission experiment: oral and subcutaneous exposure to CMt containing blood

Neither low dose (63 μ l) nor high dose (500 μ l) oral exposure to CMt-positive blood led to infection of the five recipient cats of group A (Tables 1 and 2, A III. and A IV.). These cats were subsequently inoculated subcutaneously with CMt-infectious blood: they all turned blood PCR-positive between 14 and 45 days p.i. and developed peak CMt blood loads of 10^3 to 10^5 copies/ml of blood (Table 2, A V.; Fig. 2 A to E).

In group B, four of five recipient cats subcutaneously inoculated with CMt infectious blood (Table 1, B III.) became blood PCR-positive after an average of 22 days (range: 19 - 33 days) with loads ranging from 10^3 to 10^5 /ml at peak bacteremia (Table 2, Fig. 2 F to K). One cat, Cat 7, that had received antibiotic treatment for a saliva-associated abscess 30 to 22 days prior to inoculation of

blood, remained blood PCR-negative throughout the study. Cat 7 was one of the two cats with blood-type B.

Clinical signs, i.e. fever and weight-loss were absent in the ten recipient cats inoculated with infectious blood. Anemia (PCV < 33%) was only observed in one case (Cat 1, group A), where the PCV dropped from 37 % to 28% coinciding with the peak CMt load of 9×10^5 copies/ml (Fig. 2 A). Similarly, the peak bacteremia in Cats 3 and 10 was accompanied by a slight decrease in PCV of 7% (group A, Fig. 2 C and 2 K).

4.5 Comparison of experimental infections

A significant difference in maximum CMt blood loads was observed between the amplificatory cats, cats of groups A and cats of group B (pKW = 0.0436). The intraperitoneally infected cats (amplificatory cats) showed significantly higher loads when compared to subcutaneously exposed recipient cats (groups A and B; pMWU = 0.0091; Fig. 3A). A significant difference was also found in the duration between CMt exposure and the first PCR positive result between the three groups (pKW = 0.0229) and intraperitoneally infected cats became PCR-positive earlier after CMt exposure than subcutaneously infected cats (pMWU = 0.0091; Fig. 3B). In addition, intraperitoneally infected cats had lower minimum PCV values than subcutaneously exposed cats (pMWU = 0.0182; Fig. 3C).

4.6 Correlation of anemia with CMt loads

When all CMt PCR-positive cats (n = 12) were included in the analyses, the minimum PCV values were significantly negatively correlated with maximum CMt blood loads ($r = -0.84$; $p = 0.0006$; Fig. 4A). Moreover, the minimum PCV was significantly positively correlated with duration between CMt inoculation and first PCR-positive blood result: the shorter the incubation period, the lower

the minimum PCV ($r = 0.80$; $p = 0.0017$; Fig. 4B). In addition, peak CMt blood loads were significantly negatively correlated with the incubation period ($r = -0.86$; $p = 0.0003$; Fig. 4C).

4.7 Serology

The antigen used in the Western blot assays (Mhf HspA1 fragment) was recognized by sera from cats infected with Mhf, CMhm and CMt (calculated specific band of 33.6 kDa), while sera from 18 hemoplasma-free SPF cats were all negative (Fig. 5A and 5B).

All cats used in the CMt transmission study were seronegative before CMt exposure. After CMt challenge, all 13 (amplificatory and recipient) cats became positive in Western blot analyses (Fig. 5B and 5C). Weekly investigations of serum samples demonstrated seroconversion in the majority of the cats between three and four weeks p.i. (Figs. 1 and 2).

In intraperitoneally CMt infected cats, seroconversion occurred between three to six weeks p.i. (Fig. 1). Cats remained seropositive until 13 to 15 weeks p.i.. Thereafter the bands in the Western blot assay decreased visually in intensity. In group A, four out of five cats had seroconverted three to four weeks after exposure A V. (Fig. 2A and C-E), while one cat, Cat 2 (Fig. 2B) had already seroconverted at the start of this experiment, i.e. seven weeks after oronasal inoculation of saliva (A II.). Cat 2 was the second of the two cats with blood-type B.

In group B, the four PCR-positive cats also seroconverted after CMt exposure (Table 2, B III.) between 3 and 5 weeks p.i. (Fig. 2F, H, I and K). One cat, Cat 7 (Fig. 2G)(blood-type B), that remained PCR-negative throughout the entire study, subsequently seroconverted eight weeks after a second subcutaneous inoculation of 10 μ l of blood (Table 2, B IV.).

4.8 Microscopic examination of blood smears

For none of the infected cats, independent of the CMt loads, were characteristic hemoplasma-like inclusions doubtlessly identified on blood smears by microscopy.

4.9 Antibiotic treatment

During marbofloxacin treatment CMt copy numbers decreased below detectable limits in Cats X and Y but the cats tested PCR-positive in a subsequent blood collection (Fig. 1). In contrast, after doxycycline treatment all three cats, X, Y and Z, turned persistently PCR-negative in the blood until the end of the study (167 days p.i. for Cats X and Y; 119 days p.i. for Cat Z). The nine PCR-positive recipient cats did not receive any antibiotic treatment; they all turned PCR-negative within 4-5 months and remained negative until the end of the observation period (7-10 weeks later).

5 Discussion

This is the first experimental study to investigate direct transmission routes for feline hemoplasmas, i.e. transmission via social or aggressive contact between cats, using sensitive and specific real-time PCR assays. None of the cats exposed oronasally or orally to saliva or blood of CMt-infected cats became PCR-positive for CMt in blood. The same held true for subcutaneous inoculation of saliva. In contrast, subcutaneous exposure to infectious blood led to infection.

Shedding of CMt DNA via saliva had been demonstrated in an earlier study in two experimentally infected cats (47). In addition in the present study, CMt DNA was found in saliva of all three monitored cats. Shedding started two to three weeks after exposure in all three cats. This is in accordance with our previous study (47) where we could demonstrate that shedding lasts up to 48 and 63 days. Due to the unavailability of culture assays, it cannot be determined *in vitro* whether CMt DNA in the saliva of infected cats represented viable organisms. However, according to our findings CMt-positive saliva does not pose a major risk for infection to naïve cats. Nonetheless, a seroconversion was provoked in one of the cats (Cat 2) fed with CMt-positive saliva. Thus, in the latter cat at least CMt antigens must have been transmitted via saliva and minimal CMt replication below the detection limit of the PCR or in tissues cannot be excluded. It is unlikely that the CMt loads in the saliva samples were too low to result in PCR-positivity because the same copy number given in blood was sufficient to infect cats after subcutaneous inoculation even without preexisting immunosuppression.

CMt-positive blood was found to be infectious when administered intravenously, intraperitoneally or subcutaneously (46 and present study). In contrast, oral inoculation of CMt-positive blood did not result in infection in the present study. In a very early study, transmission of 5 ml of blood from hemoplasma infected cats as determined by microscopy resulted in infection

of the recipient cats (7). However, in our opinion it seems highly unlikely that a cat would be exposed to such a large volume of infectious blood under natural conditions. We thus did not exceed 500 µl of infectious blood collected at peak bacteremia in the present study to simulate a natural/physiological way.

As a model for aggressive interactions, the subcutaneous inoculation route was chosen. All cats inoculated with as little as two drops (10 µl) of CMt-positive blood became infected. Small volumes of blood of a CMt-positive cat could be transmitted to a recipient cat via cat bite. Moreover, this volume corresponds also to that reportedly consumed by *Ctenocephalides felis* (*C. felis*) per day during hematophagous activity (5). However, experimental studies of flea transmission were not conclusive so far (50, 51) and the role of *C. felis* in the transmission of hemoplasmas is still unclear.

In accordance with our earlier study (46), CMt infection led to anemia particularly in the immunosuppressed cats. The cat that had received the highest dose of immunosuppressant (Cat Z), showed the shortest incubation period, the highest blood loads and the lowest PCV values among the three amplificatory cats. An inverse association of the CMt loads with the PCV values was found as had been reported earlier for two experimentally CMt infected cats (46).

The differences observed in the course of the CMt infections (minimal PCV, maximal CMt loads, incubation period) between the amplificatory and the recipient cats may not only be attributed to presence of immunosuppression but also to the different infection route, infection dose and age of the cats. At this point it cannot be determined whether each of these factors by itself had a significant influence on the infection. However, overall it was found that the shorter the incubation period and the higher the CMt load, the lower the PCV value. This confirms that if CMt infects a cat rapidly and vigorously, the infection leads to significant anemia.

Eight of nine cats that became PCR-positive after subcutaneous CMt exposure showed a similar infection course: the cats turned PCR-positive within two to five weeks and they all seroconverted within 5 weeks p.i. and within two weeks after PCR-positivity in the blood. Interestingly, a somewhat different course of infection was observed in two cats and remarkably, these were the only two cats with blood-type B in the present study. One cat, Cat 2, was already seropositive at the time point of the subcutaneous exposure. It had seroconverted after the oronasal exposure to infectious saliva (see above). Cat 2 became later PCR-positive (day 45) after subcutaneous exposure and developed lower CMt peak blood loads (1.4×10^3 copies/ml) compared to the other eight cats (median peak loads: 5.7×10^4 copies/ml). The second blood-type B cat, Cat 7, stayed all along PCR-negative in the blood even after repeated exposures. The cat seroconverted subsequently after the second inoculation but in the absence of PCR-positivity in the blood. While in the first cat, the oronasal exposure to CMt antigens and the induced immune response might have led to partial protection and reduced CMt loads, in the second cat the antibiotic treatment three weeks prior to the first CMt inoculation might have influenced the course of infection. Alternatively, the blood-type of the two cats, blood-type B, might have played a role in the susceptibility to CMt and infection outcome. An influence of the blood-type has been described in humans concerning other infections (11). The herein described course of CMt infection, seroconversion in the absence of detectable CMt in the peripheral blood, had not been described previously.

Microscopy of blood smears as a means of diagnosis for CMt infection cannot be recommended which is consistent with earlier findings (40, 48). We assume that the insufficient detectability might be due to the low CMt blood loads (45 and present study). They were reported to be significantly lower than Mhf blood loads (45). Even peak CMt loads (10^6 CMt/ml) calculated to only one

CMt cell in 10^3 to 10^4 erythrocytes. Thus, for reliable diagnosis of CMt infection sensitive real-time PCR must be recommended.

The herein described serological assay based on an HspA1 fragment of Mhf applied in a Western blot demonstrated seroconversion in all thirteen exposed cats after experimental infection. Using this assay, seropositive, PCR-negative animals could be identified. In the cats that had received antibiotic treatment, a decrease in band intensity in the Western blot assay was found over time, while the ten cats without treatment stayed seropositive. Antibiotic treatment might have cleared CMt from blood and tissues, while a sequestered CMt infection might have led to a continuous low level stimulation of the humoral immune response in the untreated cats. The herein described serological assay may be an excellent tool to further investigate the pathogenesis of hemoplasma infections.

In conclusion, our results indicate that social contact, such as sharing of food dishes or grooming, with CMt-positive cats does not pose a risk of infection to naïve cats. However, our hypothesis was partially confirmed insofar as direct transmission of CMt via aggressive interaction (cat bite) may occur, even if the recipient cat is exposed to a minimal volume of infectious blood.

6 Figures and Tables

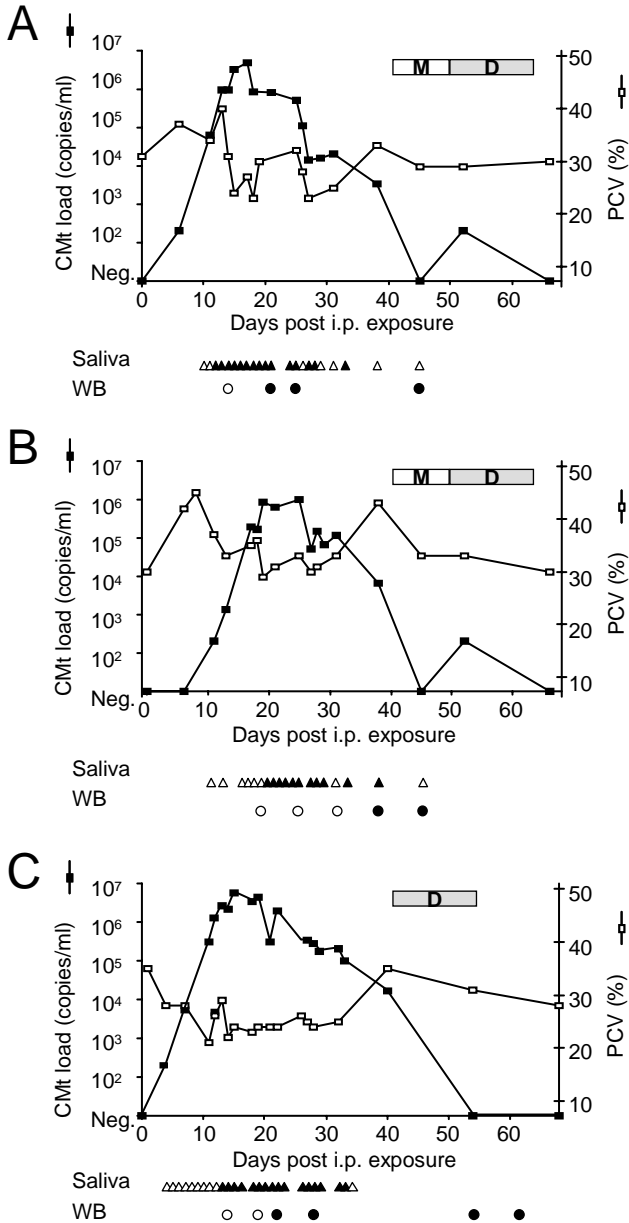


FIGURE 1. Intraperitoneal (i.p.) *Candidatus M. turicensis* (CMt) infection in the three amplificatory SPF cats, Cat X (A), Y (B), and Z (C). Kinetics of CMt blood loads (left y-axis, black squares), PCV values (right y-axis, open squares), shedding in saliva (triangle beneath x-axis) and serology results as determined by Western blot (circles beneath x-axis) throughout the time course of the CMt infection (x-axis, days p.i.). Blood loads are given in log copy numbers of DNA template per ml of blood. PCV is given as a percentage. Saliva samples were analyzed by real-time PCR: PCR-positive swabs are indicated by black triangles; negative swabs are depicted by open triangles. Western blot positive and negative serum samples are shown by black and open circles, respectively. From day 41 to day 50 p.i., Cats X and Y were treated with marbofloxacin (white square marked "M") Treatment was then switched for 14 days to doxycycline (gray square marked "D"). Cat Z was treated with doxycycline from day 41 to day 54 p.i.

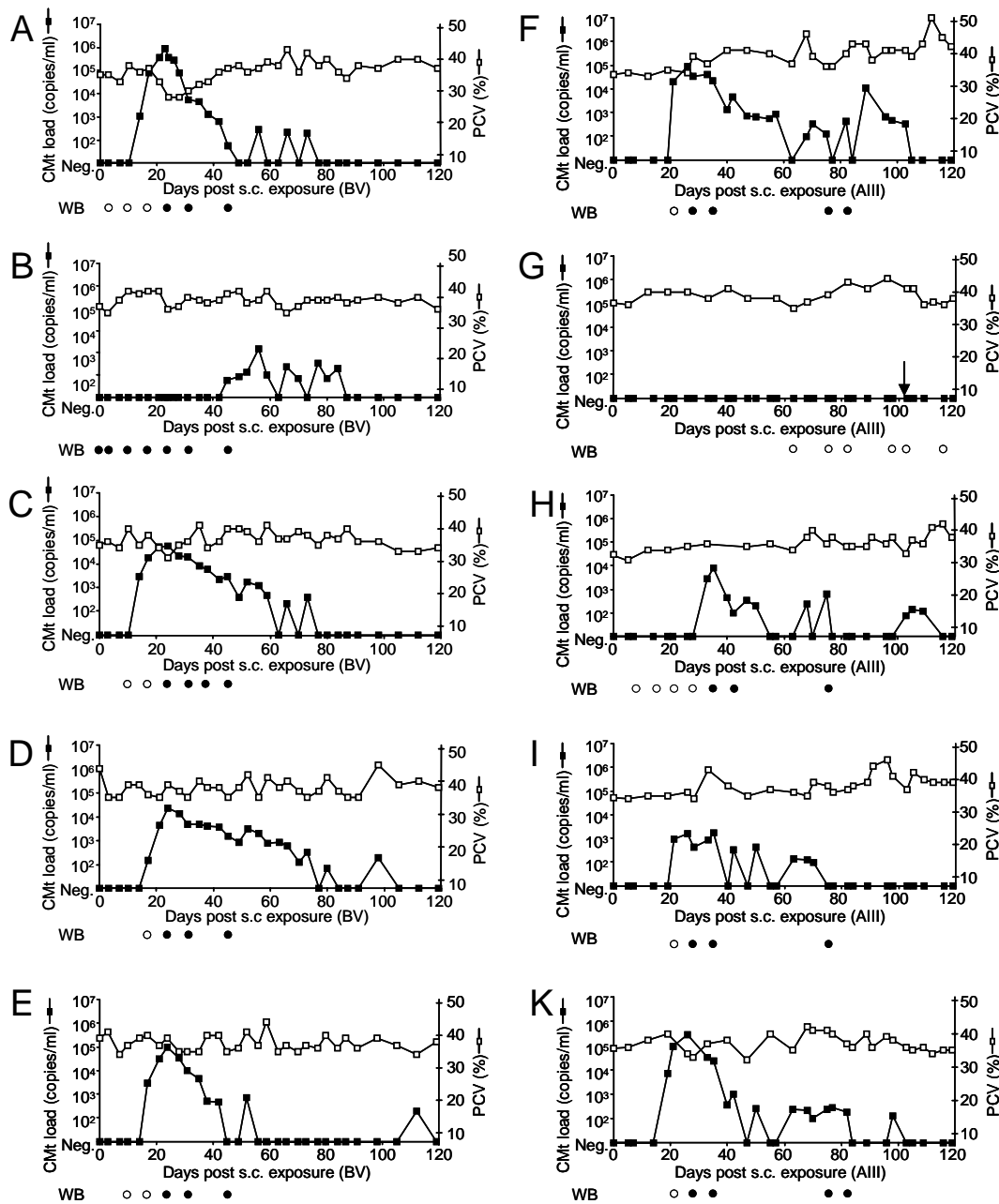


FIGURE 2. Results of transmission studies in ten SPF recipient cats after subcutaneous (s.c.) CMt inoculation experiments A V. (group A; cats 1 to 5; A to E) and B III. (group B; cats 6 to 10; F to K), respectively. Kinetics of CMt blood loads (left y-axis, black squares), PCV values (right y-axis, open squares) and serology results as determined by Western blot (circles beneath x-axis) throughout the time course of the CMt infection (x-axis, days after exposure). Blood loads are given in log copy numbers of DNA template per ml of blood. The PCV is given as a percentage. Western blot positive and negative serum samples are shown by black and open circles, respectively. Cat 7 (B) received a second subcutaneous inoculation of CMt (Table 1, exposure B IV.) at day 103 as indicated by the black arrow.

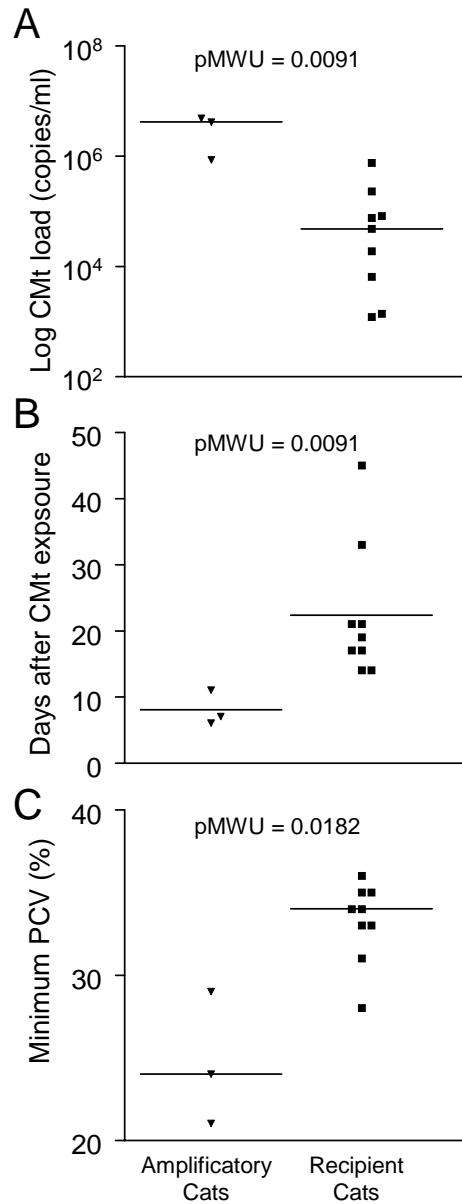


FIGURE 3. Comparison of experimental infections of CMt peak blood loads (A), duration between CMt inoculation and the first PCR-positive blood result (B), and minimum PCV values (C) in the amplificatory (intraperitoneal exposure; Cats X, Y, Z; $n = 3$) and the recipient cats (subcutaneous exposure; groups A and B; $n = 9$). Only cats that became PCR-positive were included in the analyses. Intraperitoneally infected cats had significantly higher CMt peak blood loads (A), lower minimum PCV values (B) and more rapidly became PCR-positive (C) than subcutaneously infected cats (p -values calculated by the Mann-Whitney U-test indicated in the Figure).

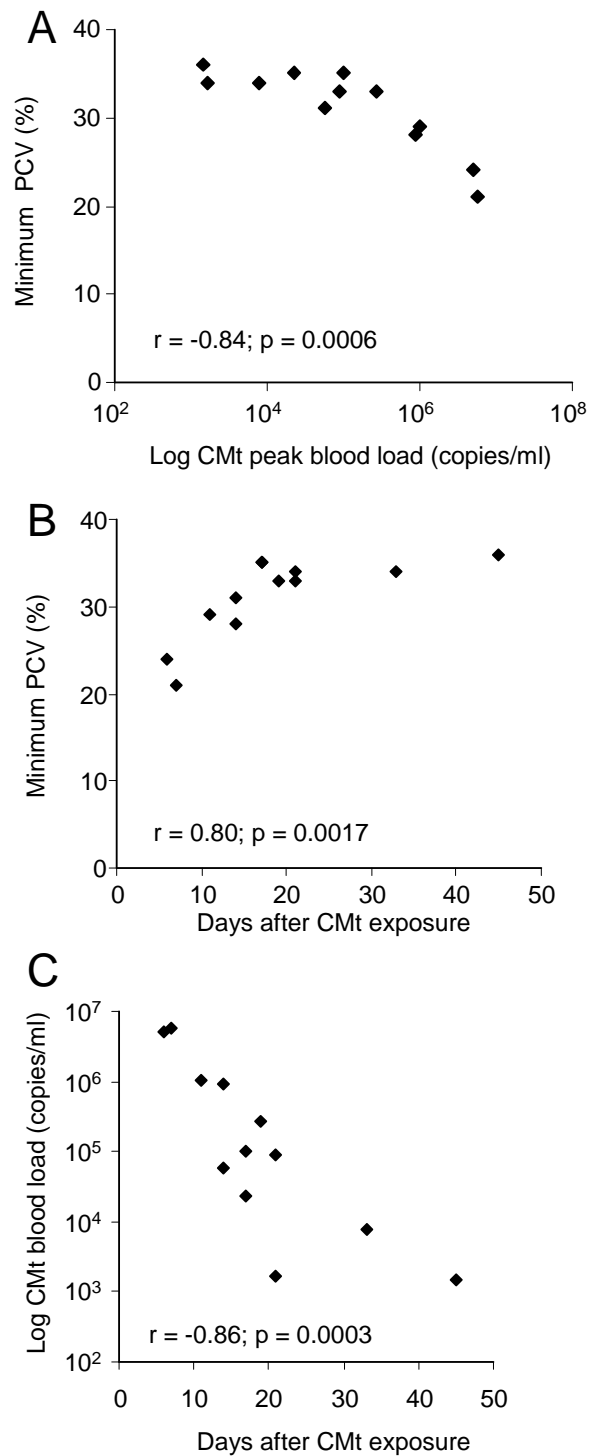
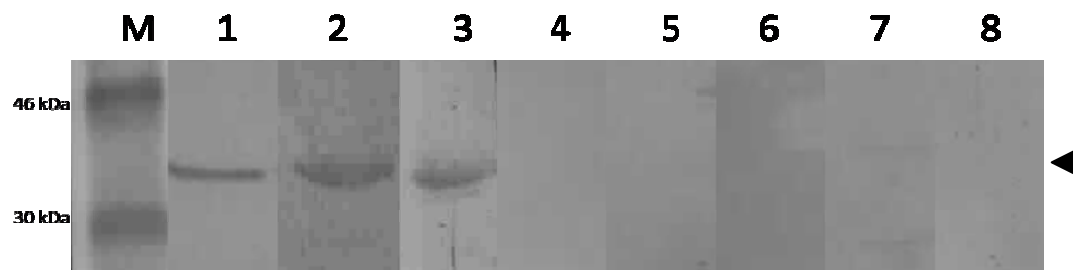
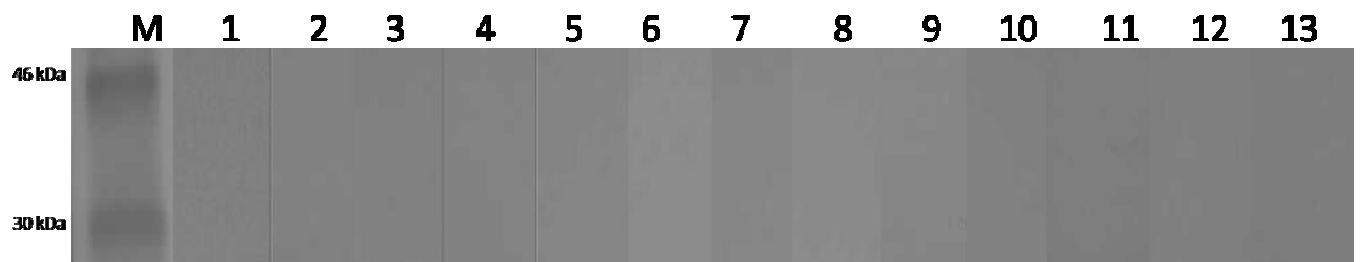


FIGURE 4. Correlation analyses of anemia and CMt loads. Minimum PCV values were negatively correlated with peak CMt blood loads (A) and significantly positively correlated with duration between CMt exposure and the first PCR-positive result (B). The peak CMt loads were significantly negatively correlated with the incubation period (C). Correlation was determined by Spearman rank correlation test (r and p values indicated in the Figure).

A)



B)



C)

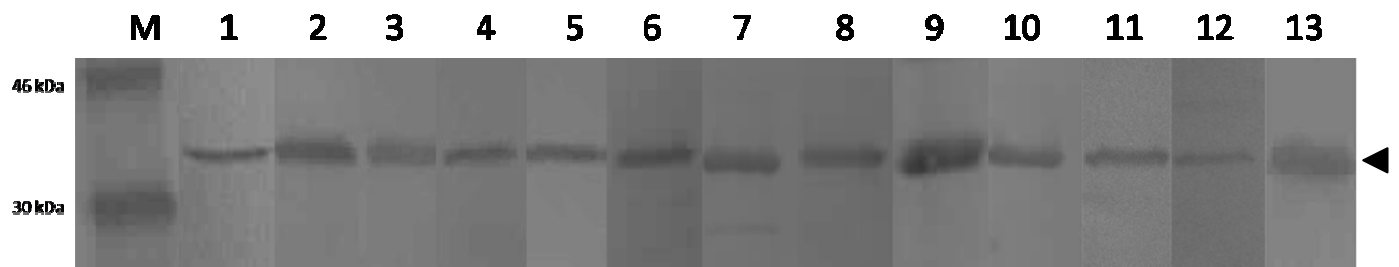


Figure 5. Western blot analyses of 16 experimentally hemoplasma infected cats and 5 SPF cats using recombinant Mhf HspA1 as antigen. A) Western blot analyses of animals infected with different hemotropic Mycoplasmas (1 = cat infected with Mhf; 2 = cat infected with CMhm; 3 = cat infected with CMt; 4 – 8 = SPF cats), B) sera of group A and B and amplificatory cats collected prior to exposure. C) sera of group A and B and amplificatory cats collected post-exposure (5 to 11 weeks after inoculation of CMt containing blood). M = Marker; stripes 1 to 5 = cats of group A; stripes 6 to 10 = cats of group B, stripes 11 to 13 = Cat X, Y, Z. Seroconversion occurred as early as three weeks p.i., but in one cat only eight weeks after CMt inoculation. Arrow at 33.6 kDa.

TABLE 1. Experimental setup of CMt transmission studies: groups and numbers of cats, experiment, protocol of immunosuppression, inoculum (infectious saliva or blood) and inoculation route and monitoring period.

Group of Cats	Experiment	Immuno-suppression	Number of cats	Inoculum (total copies of CMt)	Inoculation route	Monitoring period (days)
Group A	I. Transmission by saliva		5	2 ml saliva (8×10^2 copies)	Oronasal	31
	II. Transmission by saliva, immuno-suppression	5 mg/kg/d for 8 days; 2,5 mg/kg/d for 29 days	5 ¹	6 ml saliva (7×10^3 copies)	Oronasal	43
	III. Transmission by blood		5 ¹	63 µl blood (8×10^3 copies)	Oral	71
	IV. Transmission by blood		5 ¹	500 µl blood (4×10^5 copies)	Oral	62
	V. Transmission by blood		5 ¹	50 µl blood (6×10^3 copies)	Subcutaneous	159
Group B	I. Transmission by saliva		5	100 µl saliva (1×10^2 copies)	Subcutaneous	44
	II. Transmission by saliva, immuno-suppression	5 mg/kg/d for 8 days; 2,5 mg/kg/d for 14 days	5 ²	2 x 200 µl saliva (1×10^3 copies)	Subcutaneous	29
	III. Transmission by blood		5 ²	10 µl blood (1×10^3 copies)	Subcutaneous	99
	IV. Transmission by blood		1 ² (Cat 7)	10 µl blood (1×10^3 copies)	Subcutaneous	62

¹: same cats as used in experiment A I. ²: same cats as used in experiment B I.

TABLE 2. Outcome of CMt transmission studies (blood real-time PCR from blood and serology results) in groups and experiments described in Table 1.

Group/ Experiment	Cat 1		Cat 2		Cat 3		Cat 4		Cat 5	
	PCR	Serology	PCR	Serology	PCR	Serology	PCR	Serology	PCR	Serology
A I)	-	-	-	-	-	-	-	-	-	-
A II)	-	-	-	+	-	-	-	-	-	-
A III)	-	-	-	+	-	-	-	-	-	-
A IV)	-	-	-	+	-	-	-	-	-	-
A V)	+	+	+	+	+	+	+	+	+	+
	Cat 6		Cat 7		Cat 8		Cat 9		Cat10	
	PCR	Serology	PCR	Serology	PCR	Serology	PCR	Serology	PCR	Serology
B I)	-	-	-	-	-	-	-	-	-	-
B II)	-	-	-	-	-	-	-	-	-	-
B III)	+	+	-	-	+	+	+	+	+	+
B IV)	nt	nt	-	+	nt	nt	nt	nt	nt	nt

nt = not tested

7 References

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